Sapporo Med. J. 53 (5) 471~491 (1984)

Biochemical Characterization of the Materials Accumulated in the Alveoli of Patients with Alveolar Proteinosis

Tsugutami ONODERA
Department of Internal Medicine (Section 3), Sapporo Medical College
(Chief : Prof. A. Suzuki)

Hideki SATO and Yoko NAGATA
Department of Biochemistry (Section 1), Sapporo Medical College
(Chief : Prof. T. Akino)

Lipids and proteins of fifteen broncho-alveolar washings from five patients with alveolar proteinosis were analyzed and characterized after separating the supernatant and precipitate fractions by a brief centrifugation. The supernatant fraction contained larger quantities of proteins, which were mainly serum proteins, and had less lipids as compared to the precipitate fraction. In contrast, the precipitate fraction, i. e., water-insoluble materials, contained lipids as the major component, the majority of which was dipalmitoyl phosphatidylcholine that is known to be a main component of lung surfactant. There was only a small amount of proteins in the water-insoluble materials, but was composed mainly of proteins having molecular weights of 62,000(62 K) and 36,000(36 K).

These two proteins were glycoproteins and contained relatively high proportions of hydrophobic amino acids. The 36 K protein had the same antigenic reactivity with lung surface-active materials of human and pig, and it seemed to be localized specifically in the lung tissue. These facts suggest that the 36 K protein isolated from patient's lavages is a constituent of lung surfactant apoproteins.

These analytical findings strongly support the idea that the materials accumulated in the alveoli of patients with alveolar proteinosis are derived from the intra- and extra-cellular surfactant fractions.

The interaction between the 36 K protein and lipid mixtures was then studied in an assumption that this protein may be a subunit of lung surfactant apoproteins. The results showed that the presence of phosphatidylglycerol and cholesterol in the lipid mixtures displayed enhancing effects to the binding of liposomes to the protein, resulting in increases in the molar ratio of dipalmitoyl phosphatidylcholine to 36 K protein. However, there were no significant differences among molecular species of phosphatidylglycerol.
(Received May 14, 1984 and accepted May 28, 1984)

Key words: Alveolar proteinosis, Dipalmitoyl phosphatidylcholine, Lung surfactant, Lung surfactant apoprotein, Lung phospholipid

1 Introduction

Alveolar proteinosis is a chronic pulmonary disease characterized by an abnormal accumulation of periodate-Schiff positive amorphorus materials in the alveoli and terminal bronchioles of the lung[1]. The characteristic of lavage fluids obtained by a therapeutic broncho-alveolar lavage procedure on patients is to contain large amounts of water-insoluble materials[1,2]. These materials appear to cover the alveolar surfaces in this disease and to impair gaseous exchange. A number of studies have been made to elucidate biochemical characterization of the materials and the mechanisms by which the materials accumulate in the alveoli of patients[1-9]. However, the pathogenesis of this disease is still unknown.

Lipid chemical analyses of lavage fluids from patients with alveolar proteinosis have been made by several investigators[2-7]. The results obtained by these analyses have revealed that lavage fluids from patients with alveolar proteinosis contain much proteins and lipids as well as a higher percentage of phospholipids, particularly dipalmitoyl phosphatidylcholine.

In previous studies[2-5], the materials obtained after various centrifugations of the lung washings were analyzed. Sahu *et al.*[5] analyzed the materials sedimented by 15,000×g centrifugation after removing cells and debris. Ramirez-R and Harlan[2], Ramirez-R *et al.*[3], and McClenahan and Mussenden[4] analyzed the supernatant fraction obtained after a brief centrifugation at 300×g for 10 min. However, it is extremely difficult to separate the water-insoluble materials from cells and debris in the broncho-alveolar washings. Namely, considerable amounts of the materials seem to be sedimented with cells and debris as the water-insoluble material by a brief centrifugation. Furthermore, it is important not only to characterize the biochemical properties of the materials, but also to determine the total amounts of the materials accumulated in the alveoli in this disease. Therefore, in this study the whole lung washings were analyzed after separating the supernatant and precipitate fractions by brief centrifugation.

On the other hand, it has been reported that broncho-alveolar washings of patients with alveolar proteinosis contained relatively large amounts of characteristic proteins[8], which were identical to lung surfactant apoproteins[10]. However, there have been only a few studies regarding the profiles and properties of the proteins of broncho-alveolar washings of alveolar proteinosis[7-10]. If appreciable amounts of the characteristic proteins could be isolated from the broncho-alveolar washings, it would be an available source for the isolation of lung surfactant apoproteins, because the content of the surfactant apoproteins in the normal lung is very small. In the present study, characteristic proteins were isolated from the broncho-alveolar washings of patients, and the interaction between the characteristic protein and lipids were studied.

Therefore, the aim of the present study was to elucidate a more detailed biochemical characterization of materials accumulated in the alveoli by lipid and protein analyses on fifteen broncho-alveolar washings from five patients with alveolar proteinosis, and also to elucidate the roles of individual lipids in the forming of lipid-protein complex in the alveoli of the lung.

2 Materials and methods

2.1 Materials

2.1.1 Broncho-alveolar washings of patients with alveolar proteinosis

Fifteen broncho-alveolar washings from five patients, who were diagnosed as alveolar proteinosis clinically and/or histologically in four institutions, were analyzed in the present study: Five washings from patient 1 diagnosed in the Department of Internal Medicine(Section 3), Sapporo Medical College; two washings from patient 2 diagnosed in the Department of Internal Medicine(Devision of Respiratory Disease), Jichi Medical School; one washing from patient 3 diagnosed in the Department of Internal Medicine (Section 1), Hokkaido University Medical School; four washings from patient 4 and three washings from patient 5 diagnosed in the Department of Internal Medicine(Section 1), Asahikawa Medical College.

These broncho-alveolar lavages were taken for therapeutic purpose. The right or left lung was washed with a sterile saline solution under general anesthesia. The total influent volume used for each lung lavage ranged from 5 to 15.4 liters, with about 600 to 2,000 m*l* for each separate washing of five to ten washings. The recovery of the effluent fluid by the lavage procedure was 85.3±8.4%.

The pulmonary lavage fluid obtained was centrifuged at about 500×g for 10 min at 4°C to harvest water-insoluble particulate materials, which comprised all materials present in the alveoli in the water-insoluble form including alveolar macrophages and other cells and debris[6]. The sedimented materials by

brief centrifugation and the resultant supernatant were designated as the precipitate fraction and the supernatant fraction, respectively.

2.1.2 Alveolar wash of rat lung

Since it was difficult to obtain normal human pulmonary washings from volunteers, alveolar wash and parenchyma of rat lung were analyzed as the controls in the present study. Male Wistar rats weighing about 250 g were anesthetized with a diethylether and sacrificed by abdominal bleeding. The trachea was opened in the nech and cannulated with a polyethylene tube(Igarashi Kogyo, No. 25) connected to a syringe. The alveolar wash was obtained by introducing and withdrawing 5 m*l* of physiological saline four times. The alveolar wash was not centrifuged to remove cellular materials. The lung tissues after the alveolar washings were homogenized with saline and analyzed.

2.1.3 Lung surface-active materials and various tissue homogenates

Human lungs were taken within four hours after a patient demise from diseases other than lung diseases, and washed by saline through the main bronchus. Lung surface-active materials were then isolated from this lung washing according to the method of Frosolono *et al.*[11] Pig lung surface-active materials were also isolated by the same procedure.

Another human lung, liver, spleen and kidney tissues were taken from an autopsy. These tissues were homogenized with 0.145 M NaCl in 5 mM Tris-HCl(pH 7.4) and centrifuged at 1,000×g for 7 min. Aliquots of the supernatant fractions were used in immunological experiments.

2.1.4 Lipid materials

Dipalmitoyl phosphatidylcholine and cholesterol were purchased from Sigma Chemical Co,. Unsaturated phosphatidylcholine was isolated from egg yolk using column chromatography. Egg phosphatidylcholine(PL-100, QP company) was also used. Oligoenoic and tetraenoic phosphatidylcholines were isolated from the egg phosphatidylcholine by argentation thin-layer chromatography[13]. Dipalmitoyl, oligoenoic and tetraenoic phosphatidylglycerols were obtained by phospholipase D(Sigma Co.) treatment with glycerol from the corresponding phosphatidylcholines[14]. [Dipalmitoyl-l-^{14} C] phosphatidylcholine (specific activity, 80 mCi/mmole) was obtained from Amersham.

2.2 Lipid analysis

Lipids were extracted from the supernatant or precipitate fraction of broncho-alveolar washings, or from alveolar wash or parenchyma of rat lung, according to the method of Bligh and Dyer[15]. The amounts of phospholipid, total cholesterol, triacylglycerol and free fatty acid in the lipid extracts were determined. The amount of phospholipid was expressed as the amount of phosphorus multiplied by 25. Individual phospholipids were separated by the method of two-dimensional thin-layer chromatography described by Poorthuis *et al.*[16] as follows; silica gel G (Merck) plates prepared with 0.4 M boric acid were used. The solvent system for the y dimension was chloroform/methanol/water/conc. ammonium hydroxide (70 : 30 : 3 : 2, v/v) and for the x dimension chloroform/methanol/water (65 : 35 : 5, v/v). After chromatography, the spots on the plates were detected by charring. Individual spots were scraped off and analyzed for phosphorus.

Purified phosphatidylcholine was isolated by the combined method of column chromatography on DEAE-cellulose and preparative thin-layer chromatography as described by Akino *et al.*[17] The positional distribution of the fatty acids of the purified phosphatidylcholine was analyzed by hydrolysis with phospholipase A_2 (Naja naja, Sigma Co.)[18]. The free fatty acid and lysophosphatidylcholine prepared were separated by thin-layer chromatography. The fatty acid methylesters prepared by BF_3 methanol[19] were then analyzed by gas liquid chromatography.

The purified phosphatidylcholine was also converted to 1, 2-diacylglycerol by phospholipase C (Clostridium welchii, Sigma Co.) according to the method of Renkonen[20]. After acetylation, the 1, 2

-diacyl-3-acetylglycerol obtained was separated into molecular classes, characterized by the degree of unsaturation as described in detail by Okano *et al.*[21] The disaturated classes obtained by the argentation thin-layer chromatography were further analyzed by gas liquid chromatography for the determination of individual molecular species.

All gas chromatographies were done with a Shimadzu GC-5A gaschromatograph, equipped with a flame ionization detector, in conjunction with Shimadzu chromatopac-ElA. Fatty acid methylesters were analyzed on a 2 m×4 mm o. d. pyrex column packed with 10% diethylene glycol succinate on Shimalite W at 185° C. For analysis of 1, 2-diacyl-3-acetylglycerols, a 50 cm×4 mm o. d. pyrex column was packed with 1% OV-1 on Gaschrom Q. The oven was operated isothermally at 285° C

Lipid phosphorus was determined by the method of Bartlett[22]. Glycerol was determined by the method of Van Handel and Zilversmit[23]. Total cholesterol, triacylglycerol and free fatty acid were determined by the enzyme method using commercial kits (Kyowa Medex).

2. 3 Protein analysis

The amount of protein in the supernatant and precipitate fractions of the broncho-alveolar washings was determined by the method of Lowry *et al.*[24] Aliquots of both fractions were dissolved in 0.2% sodium dodecyl sulfate (SDS)-0.1% 2-mercaptoethanol-40 mM phosphate buffer (pH 7.0) and separated by SDS -polyacrylamide gel electrophoresis according to the method of Weber and Osborn[25]. The gels were either stained for protein with Coomassie blue or for glycoprotein with periodate-Schiff reagent[26]. Molecular weight estimations for the peptides on the gels were made with phosphorylase b (M. W. 94,000), bovine serum albumin (M. W. 67,000), ovalbumin (M. W. 43,000), carbonic anhydrase (M. W. 30,000), trypsin inhibitor (M. W. 20,100) and α-lactalbumin (M. W. 14,400) as the standards.

The isolation of 36,000 (36 K) and 62,000 (62 K) dalton proteins from the precipitate fraction was performed as follows: Most of lipids in the precipitate fraction were extracted with n-butanol/ethanol (6 : 1, v/v)[12], and the proteins obtained after lipid removal were solubilized in 5 mM This-HCl buffer (pH 7.4) containing 5 M urea and 0.02% 2-mercaptoethanol and passed through a column of Blue Sepharose CL 6B to remove the albumin. The proteins eluted from Blue Sepharose column were applied to a column (2.5×80 cm) of Sephadex G-200 equilibrated with the same buffer. The elution of peptides was done with the equilibrating buffer. The major peaks were checked for purity by SDS-polyacrylamide gel electrophoresis as described above. Main peak fractions containing 62 K or 36 K protein were further fractionated through the DEAE-cellulose column, employing a linear NaCl gradient (0-1.0 M) in the same buffer. Thereafter, a gel filtration by Sephadex G-200 column was performed to purify 36 K or 62 K protein from fractions mainly containing 36 K or 62 K protein obtained by DEAE-cellulose column chromatography.

Preparative SDS-polyacrylamide slab gel electrophoresis was also carried out to isolate 36 K and 62 K proteins. Proteins of the precipitate fraction were applied on the gel as a band (100-150 μg protein/cm of 2 mm thick of 10% polyacrylamide gel). After the electrophoresis, the proteins were visualized by staining with Coomassie blue. Bands corresponding to each peptide were cut out and sliced. Each protein was then separated from the staining reagent by an electrophoretic elution procedure as described by Hunkapiller *et al.*[27] Thereafter, SDS was removed by dialysis.

2. 4 Immunological analysis

36 K protein isolated from the slab gels was dialyzed against phosphate buffered saline (PBS) for 3 days, changing the PBS every day. The suspension containing 36 K protein was emulsified with an equal volume of Freund's complete adjuvant. Aliquots of 1 m*l* of the emulsion, containing 100 μg of 36 K protein, were inoculated intraperitoneally into BALB/c female mice, 8 weeks old. A month later, a booster injection of 36 K protein in PBS was carried out intraperitoneally. The animals were bled and the antisera collected 3 days after the last injection. Antisera to LP (lavage precipitate fraction) proteins which were

found in the precipitate fraction of lavages of patients with alveolar proteinosis and contained predominantly 62 K and 36 K proteins, was also prepared in the same manner as described above.

Specificities of antisera to 36 K protein and LP proteins were determined by dot-immunobinding assay as described by Hawkes *et al.*[28] and Esen *et al.*[29]

2.5 Formation of lipid-protein complex

2.5.1 Labeling of 36 K protein with $Na^{125}I$

The protein of 36,000 molecular weight, isolated from the lavage precipitate of patients with alveolar proteinosis, was labeled with $Na^{125}I$ by the procedure of Hunter and Greenwood[30], using chloramine-T.

2.5.2 Preparation of liposomes

The lipid mixtures used for studies on formation of lipid-protein complex are illustrated in Table 1. The lipid mixtures were dispersed in an aqueous buffer as described by King and MacBeth[31] in the following manner. Lipid mixtures containing tracer amounts of ^{14}C-dipalmitoyl phosphatidylcholine were dissolved in ethanol at a concentration of about 25 mg/m*l*. The ethanol-dissolved lipids were rapidly injected with the aid of a Hamilton syringe into a buffer warmed at 45° C, the volume of which was greater than ten-times the volume injected ethanol, and then sonicated using a Branson sonicator for 1 min. The buffer solution used was 0.1 M NaCl/3 mM $CaCl_2$/5 mM Tris-HCl, pH 7.4 (buffer A).

Table 1 *Composition of lipid mixtures used for the formation of lipid-protein complex.*

Mixture	DPPC	DPPG	UPC	Cholesterol
1	63	9	19	9
2	77	13	0	10
3	86	14	0	0
4	64	0	27	9

Values are expressed as mole %.
DPPC, dipalmitoyl phosphatidylcholine (containing ^{14}C-palmitate); DPPG, dipalmitoyl phosphatidylglycerol; UPC, unsaturated phosphatidylcholine isolated from egg yolk.

2.5.3 Formation of lipid-protein complex using 36 K protein and liposomes

The 36 K protein dissolved in 5 mM Tris-HCl (pH 7.4) was mixed with the liposomes dispersed in the buffer A. In most experiments, the interaction was carried out in the double labels with ^{125}I-36 K protein and liposomes containing ^{14}C-dipalmitoyl phosphatidylcholine. The single label experiments with ^{125}I-36 K protein or ^{14}C-dipalmitoyl phosphatidylcholine were also carried out. The protein was added to the lipid suspensions to give a final concentration of protein at 25 μg/2m*l* which corresponds to 0.7×10^{-3} μmole/2m*l* calculated on the basis of a monomeric molecular weight of 36,000.

Most experiments were carried out as illustrated in Fig. 1. The ^{125}I-36 K protein (25 μg) and the liposomes (1 μmole lipid) containing ^{14}C-dipalmitoyl phosphatidylcholine in 2 m*l* of the buffer A were mixed in a teflon tube at 37° C by sonication (50 watt, 1 min 2 times), and kept at 37° C for 10 min. The mixture was dialyzed against about 2000 volumes of buffer A at 25° C for 20-25 hrs., and centrifuged at 18,000×g for 60 min at 20° C. The pellet obtained by the centrifugation was suspended in 0.5 m*l* of buffer A warmed at 20° C. The lipid-protein mixture was layered over 3.6 m*l* continuous gradient ranging from 4 to 17%, poured over a 0.3 m*l* cushion of 40% sucrose. The samples were centrifuged for 120 min at 20,000 rpm in a Hitachi RPS 56 T swinging bucket roter. Temperature was maintained at 20° C throughout the period of centrifugation. After the centrifugation, each 0.18 m*l* of the samples was taken from the top to the centrifuged tube, transferred directly to scintillation counting vials, and the radioactivity was determined in 10 m*l* of ACS-II (Amersham) under a program of the radioactivity determination on ^{125}I and ^{14}C double labels as described below.

2.6 Analytical methods

2.6.1 Amino acid analysis

Amino acid analysis was performed by the method of Spackman[32] on a Hitachi automatic analyzer.

Lipid mixture (1 μmole) containing ^{14}C-DPPC dispersed in 1 m*l* of 0.1 M NaCl, 5 mM Tris-HCl (pH 7.4) containing 3 mM $CaCl_2$ (buffer A)
↓
The suspension was vigorously stirred with Vortex mixer at room temperature
↓
Purified ^{125}I-36 K protein (25 μg) was added
↓
The mixture was sonicated, kept at 37° C for 10 min and dialyzed against buffer A for 20–25 hrs. centrifuged at 18,000 xg for 1 h. at 20° C
↓
The pellet was suspended in 0.5 m*l* of the buffer A warmed at 20° C
↓
The sample was layered over sucrose continuous density gradient (3.6 m*l* of linear sucrose density gradient from 4 to 17%, poured over 0.3 m*l* cushion of 40% sucrose) centrifuged at 40,000 xg for 2 hrs. at 20° C
↓
Each fraction (0.18 m*l*) was collected from the top of the gradient
↓
Radioactivity counting (^{125}I and ^{14}C double labels)

Fig. 1 Experimental procedures for the formation of lipid-protein complex.
DPPC: dipalmitoyl phosphatidylcholine

Before analysis, the proteins were hydrolyzed in 6 N HCl in a sealed tube at 110° C for 24 hours.

2. 6. 2 Determination of radioactivity doubly labeled with ^{125}I and ^{14}C

The radioactivity was determined with a liquid scintillation spectrometer (Beckmann LS 9000). The windows of the scintillaiton counter were set so that there was no spillover of ^{125}I counts into the ^{14}C channel, and less than 40% spillover of ^{14}C counts into the lower energy window. When the ratio of ^{125}I and ^{14}C activities was about 1 to 1, more accurate values were obtained in this program.

3 Results

3.1 Contents of phospholipid and protein

Broncho-alveolar lavages of each of the patients produced considerable amounts of water-insoluble particulate materials. Therefore, the brief centrifugation was carried out to separate the water-insoluble materials. Fig. 2 shows a typical profile of protein and phospholipid contents in the lung lavage fractions of a patient obtained by a therapeutic lung lavage procedure using 1.0–2.0 *l* for each separate washing. The rapid declining curve of protein and phospholipid in the successive lavage fractions appears to show that most of the materials present in the alveolar lumen could be removed by this lavage procedure.

The amounts of phospholipid and protein in the washings of patients with alveolar proteinosis, which were obtained using from 5 to 15.4 *l* of saline for therapeutic purposes, are shown in Table 2. The amounts of phospholipid and protein in the total washings were 2.26±1.44 and 17.0±7.9 g, respectively. The phospholipid was found to be in much higher amounts in the precipitate fraction (84.1%) than in the supernatant fraction (15.9%). In contrast, protein was found to be in significantly higher amounts in the supernatant fraction (85.3%). The ratio of phospholipid to protein in the precipitate fraction was 0.76, which was similar to that (0.70) in alveolar wash of rat lung.

	1	2	3	4	5	6	7	8	9	10
Influent volume (l)	1.00	1.20	1.30	1.40	1.50	1.60	1.70	1.80	1.90	2.00
Effluent volume (l)	0.60	0.95	1.00	1.15	1.12	1.25	1.35	1.35	1.45	1.60

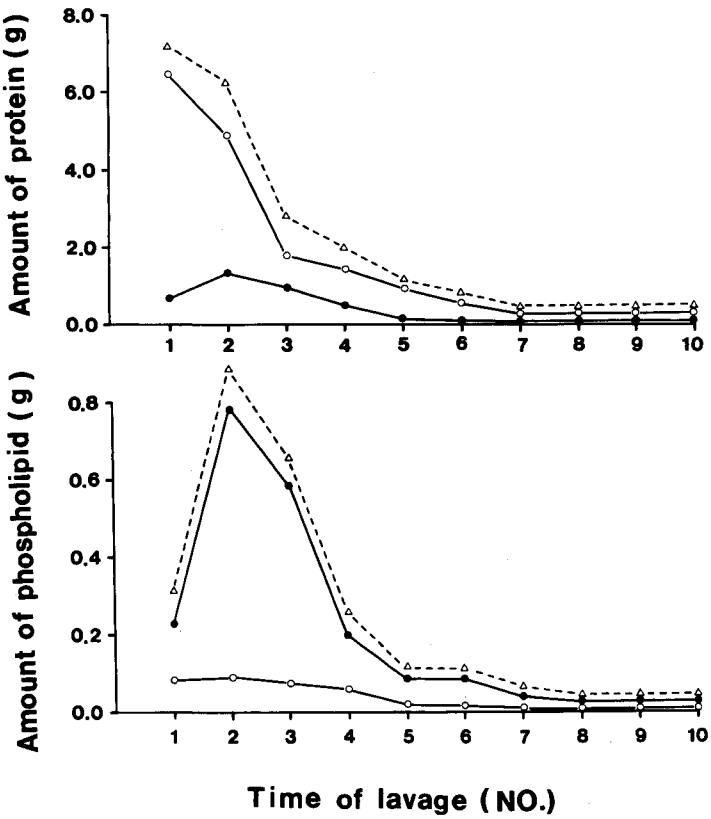

Fig. 2 A typical profile of amounts of protein and phospholipid obtained by successive broncho-alveolar lavages of a patient with alveolar proteinosis.
△·····△ : total lavage fluid (supernatant fraction plus precipitate fraction)
○·····○ : supernatant fraction
●·····● : precipitate fraction

Table 2 *Contents of protein and phospholipid in broncho-alveolar washings from patients with alveolar proteinosis.*

		Protein	Phospholipid	Phospholipid/ Protein
Alveolar proteinosis (n=15)	Supernatant	14.5 ±6.7g (85.3)	0.36±0.24g(15.9)	0.03
	Precipitate	2.5 ±1.8 (14.7)	1.90±1.29 (84.1)	0.76
	Total	17.0 ±7.9	2.26±1.44	0.16
		mg/g wet tissue		
Rat lung (n=4)	Parenchyma	70.5 ±4.2	14.0 ±1.9	0.20
	Alveolar wash	1.28±0.12	0.90±0.23	0.70

Values in parentheses represent the percent distribution between supernatant and precipitate fractions.

3. 2 Characteristics of phospholipids

Composition of lipid class in the broncho-alveolar lavages is given in Table 3. Phospholipid was the major component of the lipid class in both fractions. It constituted about 85% of the total lipid in the lavage sediment and about 69% in the supernatant fraction. The phospholipid contents in the lavages were higher than that in rat lung alveolar wash. Phosphatidylcholine constituted the largest component of the total lipid in the lavages, but the proportion of phosphatidylcholine was found to be somewhat different between the two fractions. Phosphatidylcholine comprised about 60% of the total phospholipid in the precipitate fraction, but in the supernatant fraction sphingomyelin and phosphatidylcholine were the main phospholipids. Compared to the phospholipid composition of rat lung alveolar wash, much sphingomyelin and less phosphatidylcholine were found to be in the precipitate fraction. Since the materials accumulated in the alveoli of patients with this disease had been exposed to oxygen for a long time, it is possible that phosphatidylcholine having unsaturated fatty acids may have in part been oxidized. Sphingomyelin, on the other hand, seems to be more stable against oxygen attack than phosphatidylcholine.

Fatty acid profiles of 1- and 2-positions of phosphatidylcholine are shown in Fig. 3. The fatty acids of phosphatidylcholine in the precipitate fraction were highly saturated at both positions, that is, palmitic acid was 89% at the 1-position and 73% at the 2-position. Phosphatidylcholine in the supernatant fraction, however, contained less palmitic acid at both positions, and more unsaturated fatty acids were found at the 2-position. It was also noted that fatty acids at both positions of phosphatidylcholine in broncho-alveolar lavages were much more saturated than those in rat lung alveolar wash. This finding suggests that the phospholipids present on the alveolar surface in this disease are more oxidized and lose more unsaturated fatty acids, probably due to oxygen exposure in the alveoli over a long period of time.

Data on molecular species of phosphatidylcholine further support the presence of highly saturated phosphatidylcholine in the alveoli of patients with alveolar proteinosis (Fig. 4). The predominant

Table 3 *Lipid composition of broncho-alveolar washings.*

	Alveolar proteinosis		Rat lung	
	Supernatant	Precipitate	Parenchyma	Alveolar wash
Lipid class (weight %)				
Triacylglycerol	0.4±0.3	0.4±0.3	20.1	8.2
Free fatty acid	14.3±5.3	2.0±1.4	2.2	1.0
Total cholesterol	16.2±2.6	12.9±5.9	15.9	12.8
Phospholipid	69.1±5.5	84.6±6.8	61.8	78.0
Phospholipid class (mole %)				
Phosphatidylcholine	40.6±9.0	60.4±4.8	46.2	79.1
Phosphatidylethanolamine	9.8±5.5	4.2±1.2	24.2	5.1
Phosphatidylglycerol	4.3±1.5	6.8±1.8	3.4	9.0
Phosphatidylinositol	5.4±2.2	6.0±2.1	6.4	1.2
Phosphatidylserine	5.3±2.0	3.1±1.6	6.2	1.1
Sphingomyelin	22.9±7.8	9.3±2.6	11.0	3.9
Lysophosphatidylcholine	3.5±2.8	2.9±1.6	1.6	tr
Cardiolipin	2.6±1.5	1.6±0.8	0.4	tr
Lyso-bis-phosphatidic acid	5.7±2.3	5.9±2.9	0.6	0.6

Distribution percents of lipid class of alveolar proteinosis are means±S. D. (n=6).
Values of phospholipid class composition of alveolar proteinosis are means±S. D. (n=15).
Values of rat lung are averages of duplicate analyses.

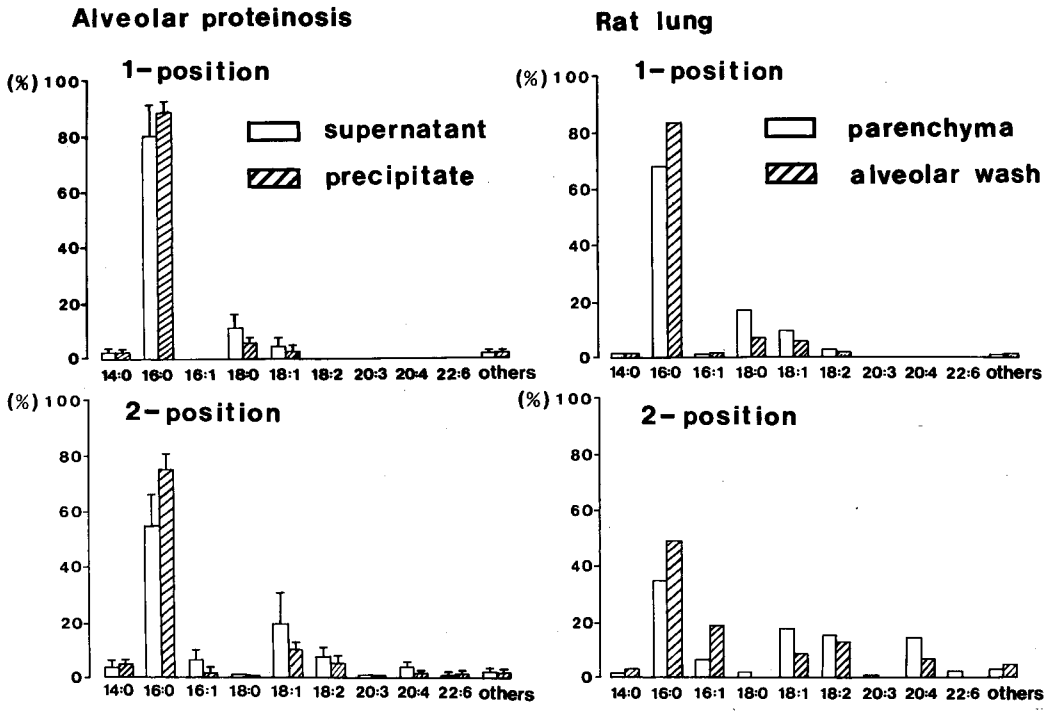

Fig. 3 Fatty acid composition of phosphatidylcholine of broncho-alveolar washings from patients with alveolar proteinosis. Data of alveolar proteinosis are means of 15 cases. Vertical lines express standard deviations. Data of rat lung are means of 2 cases.

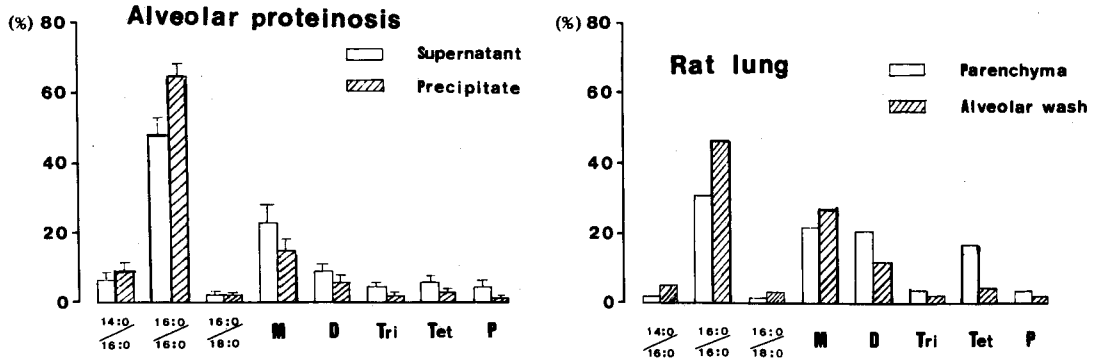

Fig. 4 Composition of molecular species of phosphatidylcholine of broncho-alveolar washings from patients with alveolar proteinosis. Data of alveolar proteinosis are means of 5 cases. Vertical bars express standard deviations. Data of rat lung are means of 2 cases.

14 : 0/16 : 0 : myristoyl-palmitoyl species
16 : 0/16 : 0 : dipalmitoyl species
16 : 0/18 : 0 : palmitoyl-stearoyl species
M: monoenoic species, D: dienoic species, Tri: trienoic species, Tet: tetraenoic species, P: polyenoic species (pentaenoic plus hexaenoic species)

molecular species was a dipalmitoyl type in both fractions of lavages, comprising 68% in the precipitate fraction and 50% in the supernatant fraction. It was also noted that the dipalmityl phosphatidylcholine

content in the precipitate fraction was higher than that in rat lung alveolar wash.

These findings on the phospholipid analyses demonstrate that the predominant phospholipid accumulated in the alveoli of the patients is a dipalmitoyl phosphatidylcholine, which is known to be a main component of lung surfactant.

3. 3 Major proteins in the supernatant and precipitate fractions

As shown in Table 2, the protein content of the broncho-alveolar washings was much higher in the supernatant fraction than in the precipitate fraction. SDS-polyacrylamide gel electrophoresis revealed that proteins of the supernatant fraction were very similar to serum proteins, while those of the precipitate fraction were distinctly different from those in the supernatant fraction (Fig. 5). Although protein profiles

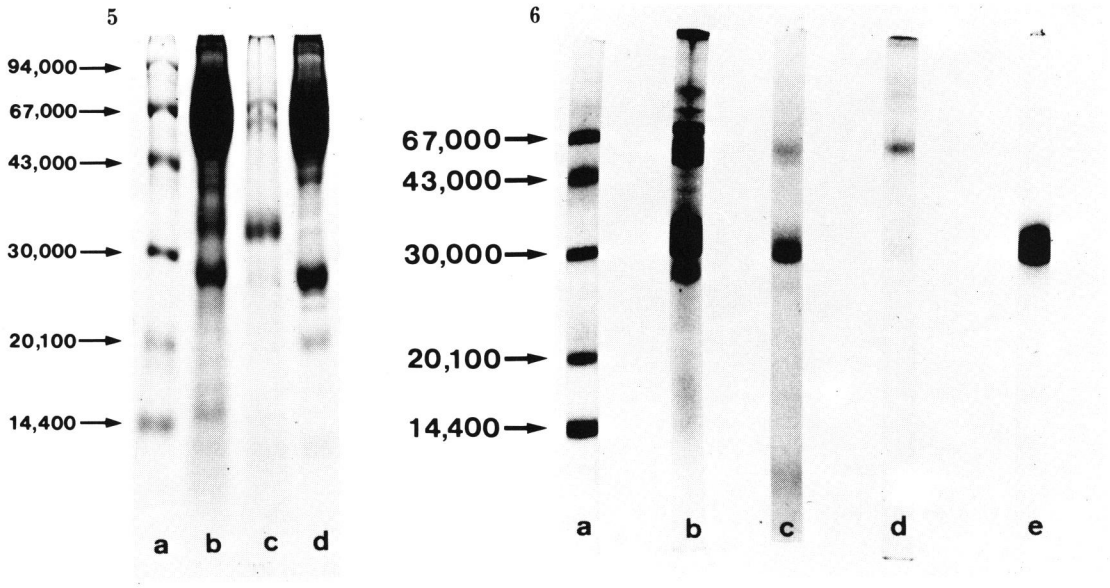

Fig. 5 SDS-polyacrylamide gel electrophoresis of proteins of broncho-alveolar washings from patients with alveolar proteinosis. The supernatant and precipitate fractions containing about 40 μg of protein were dissolved in 2% SDS-0.1% 2-mercaptoethanol (pH 7.0), and applied to SDS-10% polyacrylamide gels. Protein bands were stained with Coomassie blue.

a: protein standards; phosphorylase b (M. W. 94,000), bovine serum albumin (M. W. 67,000), ovalbumin (M. W. 43,000), carbonic anhydrase (M. W. 30,000), soybean trypsin inhibitor (M. W. 20,100) and α-lactalbumin (M. W. 14,400)

b: supernatant fraction

c: precipitate fraction

d: human serum

Fig. 6 SDS-polyacrylamide gel electrophoresis of proteins in the precipitate fraction of broncho-alveolar washings from patients with alveolar proteinosis, and proteins isolated by column chromatographies on Sephadex G-200 and DEAE-cellulose.

The electrophoresis was performed according to the method of Weber and Osborn[25].

a: protein standards as described in Fig. 5

b: delipidated proteins of precipitate fraction stained with Coomassie blue

c: delipidated proteins of precipitate fraction stained with periodate-Schiff's reagent

d: 62,000 molecular weight protein isolated from the lavage precipitate fraction

e: 36,000 molecular weight protein isolated from the lavage precipitate fraction

of the precipitate fraction in SDS-polyacrylamide gel electrophoresis were somewhat different among lavage samples, major bands were proteins corresponding to molecular weights of 62,000 (62 K) and 36,000 (36 K) as determined on the gels. The 36 K protein band was detected as a broad band on the gels. Other protein bands corresponding to molecular weights of 69,000 (69 K) and 28,000 (28 K) were also detected in various intensities among different samples (Fig. 5-c, Fig. 6-b).

These findings demonstrate that the great majority of proteins accumulating in the alveoli of patients with alveolar proteinosis are serum proteins, and the proteins in the water-insoluble materials found in this disease are proteins other than serum proteins, one of which, i. e., 36 K protein, has an identical molecular weight with a main component of apoproteins of lung surface-active materials as reported previously[36,37].

3. 4 Isolation of 36 K and 62 K proteins

The precipitate fraction of the broncho-alveolar lavages was delipidated by butanol-ethanol method. After the extraction of most lipids with butanol-ethanol, the proteins were separated by SDS-polyacrylamide gel electrophoresis which revealed that the proteins contained three major bands (Fig. 6-b). The molecular weights of the three proteins determined on the gels were 69,000 (69 K), 62,000 (62 K) and 36,000 (36 K) daltons, and the latter two bands contained carbohydrate (Fig. 6-c).

After removing most of albumin by Blue sepharose CL 6B column chromatography, the remaining proteins were separated by gel filtration on Sephadex G-200 column. The results are shown in Fig. 7. Three major peaks were obtained, that is, the first peak (A and A') eluted in a void volume which contained 69 K and 62 K proteins, and the second (B) and third (C) peaks containing 36 K and 15 K proteins, respectively, as major components. The 62 K and 36 K proteins were then separated by DEAE-cellulose column chromatography (Fig. 8). The 62 K rich (A'_2) and 36 K rich (B_2) fractions were still contaminated with small amounts of other proteins. Therefore, they were further purified by Sephadex G-200 column chromatography. Finally, the 36 K protein showed only one band on the gel of SDS-polyacr-

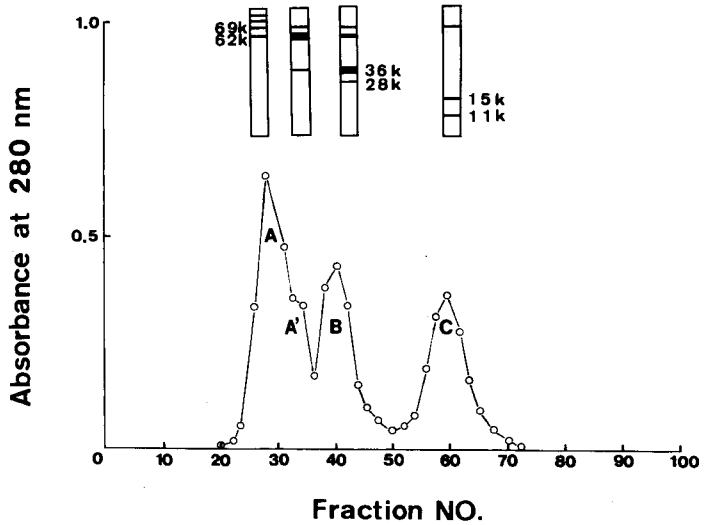

Fig. 7 Separation of proteins in the precipitate fraction of broncho-alveolar washings by gel filtration on Sephadex G-200 column in 5 mM Tris -HCl buffer (pH 7.5) containing 5 M urea and 0.02% 2-mercaptoethanol. The SDS-polyacrylamide gel electrophoretic profiles of the peak fractions A, A', B and C are shown in the insets.

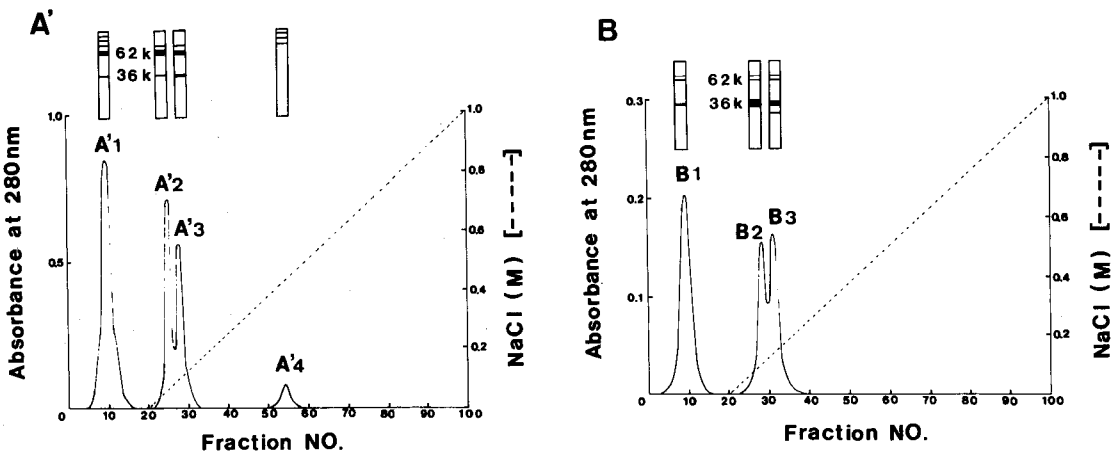

Fig. 8 DEAE-cellulose column chromatographies of proteins in the peak fractions (A′ and B) obtained by Sephadex G-200 column chromatography of the sedimented proteins of broncho-alveolar washings. The DEAE-cellulose column chromatographies were carried out with a linear NaCl gradient (0-1.0 M) in 5 mM Tris-HCl buffer (pH 7.4) containing 5 M urea and 0.02% 2-mercaptoethanol. The SDS-polyacrylamide gel electrophoretic profiles of the peak fractions are shown in the insets.
A′: A′ fraction (tube No. 32-35) in Fig. 7
B : B fraction (tube No. 36-48) in Fig. 7

ylamide gel electrophoresis (Fig. 6-e). The yield of the 36 K protein was about 10% of the total protein of the original 500×g precipitate fraction of broncho-alveolar lavages, although it varied considerably among the samples. The 62 K protein isolated, as described above, was still contaminated with small amounts of other proteins remaining at the origin of the gel (Fig. 6-d).

The 36 K and 62 K proteins were also isolated by preparative SDS-polyacrylamide slab gel electrophoresis. The amino acid constituents of each protein isolated by this method were analyzed (Table 4). The percentage of hydrophobic amino acids of 36 K and 62 K proteins was 68.4% and 64.7 %, respectively. The 36 K protein contained hydroxyproline, suggesting that it is a collagen-like glycoprotein, but the 62 K protein did not contain this amino acid. Distinct differences betwen the two proteins were also observed in the contents of glycine, cystine and tyrosine. The 62 K protein did not contain cystine and tyrosine. The glycine content of the 36 K protein was about 1.5 fold of the 62 K protein. These amino acid profiles obtained in the present study were somewhat different from those reported by Bhattacharyya *et al.*[9]

Table 4 *Amino acid composition of proteins isolated from broncho-alveolar washings.*

	36 K protein	62 K protein
	Residure per 1,000 amino acids	
Lys	37.1	59.4
His	17.9	20.4
Arg	51.9	47.8
Asp	113.2	96.7
Ser	52.8	78.6
Thr	46.2	55.0
Glu	96.2	128.5
Pro	60.8	60.0
Gly	211.8	142.4
Ala	62.9	77.8
1/2-Cys	12.5	N. D
Val	32.7	63.8
Met	13.1	10.5
Ile	37.8	34.7
Leu	63.7	85.9
Tyr	23.4	N. D
Phe	34.0	38.5
Hyp	32.0	N. D
Hyl	N. D	N. D

Values are means of duplicate analyses.
N. D; not detected

3.5 Immunological characterization of 36 K protein

The immunological properties of antisera developed against 36 K protein and LP proteins isolated from the lavage precipitate fraction after removal of albumin, were studied. When dot-immunobinding assay was employed, antisera to 36 K protein or LP proteins reacted intensely with lung surface-active materials of human or pig as well as 36 K protein and LP proteins, but both antisera did not react with human serum albumin (Fig. 9). The tissue distribution of 36 K protein and LP proteins was studied by dot -immunobinding assay using antisera developed against 36 K protein or LP proteins. The results are shown in Fig. 10. The reactivities of extracts of liver, spleen and kidney were almost the same between normal mouse serum and anti-36 K protein serum or anti-LP protein serum, indicating that these tissue extracts did

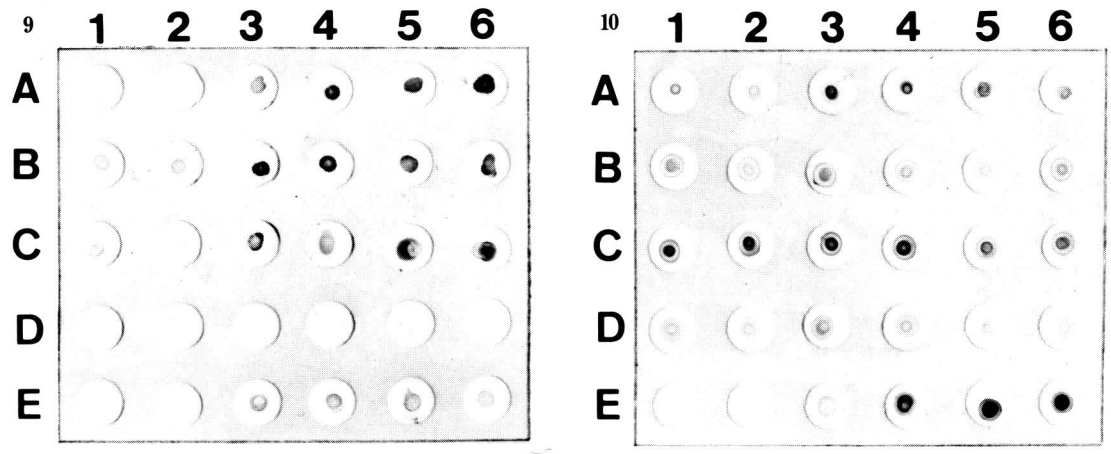

Fig. 9 Dot immunobinding assays for antibodies developed against 36 K protein and LP (lavage precipitate fraction) proteins.
1 and 2: normal mouse serum
3 and 4: mouse anti-36 K protein serum
5 and 6: mouse anti-LP protein serum
A: Surface active materials isolated from human lung washings
B: LP proteins isolated from lung washings of patients
C: 36 K protein isolated from lung washings of patients by preparative SDS-polyacrylamide gel electrophoresis
D: human serum albumin (Sigma)
E: surface active materials isolated from pig lung washings
0.2-0.4 μg proteins were used in A, B, C and E, but in D 1.0 μg protein was used.

Fig. 10 Tissue distribution of 36 K protein and LP (lavage precipitate fraction) proteins by dot -immunobinding assays.
1 and 2: normal mouse serum
3 and 4: mouse anti-36 K protein serum
5 and 6: mouse anti-LP protein serum
A: human lung homogenate
B: human liver homogenate
C: human spleen homogenate
D: human kidney homogenate
E: surface active materials isolated from human lung washings
Samples of A, B, C, D and E were suspended in 1% Triton X-100 and then dot -immunobinding assays were carried out. 5-10 μg proteins were used in A, B, C and D, but in E 0.25 μg protein was used.

not react with the antisera to 36 K protein and LP proteins, although some of the extracts gave a non-specific reaction with the sera. In contrast, the lung homogenate reacted with antisera to 36 K protein or LP proteins, but it did not react distinctly with normal mouse serum. This reactivity of the lung homogenate was very similar to that of human lung surface-active materials. These results indicate that 36 K protein seems to be localized in the lung, but not in any of the other tissues tested. Therefore, these immunological studies of 36 K protein isolated from patient's lavages demonstrate that the 36 K protein has the same antigenic determinants as lung surface-active materials of human or pig, and it may exist specifically in the lung. Furthermore, these facts strongly suggest that the 36 K protein in patient's lavages is a constituent of lung surfactant apoprotein, since it has been reported that surface-active materials of normal lung washings contain a protein having the same molecular weight as the main constituent[36,37].

3.6 Formation of lipid-protein complex

The interaction of 36 K protein, which was isolated from the precipitate fraction of patient's lavages, and lipids corresponding to lung surfactant lipids were then studied. The lipid mixture 1 was constituted to resemble the composition of the principal lipid in surfactant. ^{125}I-36 K protein (25 μg) was sonicated with each 1 μmole of these lipid mixtures containing ^{14}C-dipalmitoyl phosphatidylcholine. After dialysis of the mixture, the recovery of the ^{125}I-radioactivities in pellets obtained from 18,000×g centrifugation was almost the same among the lipid mixtures (Table 5). However, there were significant differences in the yield of ^{14}C-activity among the mixtures. The yield of ^{14}C-activity in the 18,000×g pellet was 70% in mixture 1, while that in mixtures 3 and 4 was about 21%, indicating that the unbounded ^{14}C-dipalmitoyl phophatidylcholine in mixtures 3 and 4 might be lost in the 18,000×g supernatant or during the dialysis.

Table 5 *Yield of radioactivity in pellets obtained at 18.000×g centrifugation after the sonication followed by the dialysis of ^{125}I-36 K protein and various lipid mixtures containing ^{14}C-dipalmitoyl phosphatidylcholine.*

Lipid mixture	Yield (%)	
	^{125}I-activity	^{14}C-activity
1	69.8	70.5
2	62.1	50.9
3	67.5	21.5
4	68.5	21.4

The yield was calculated from ^{125}I-and ^{14}C-radioactivities in the original mixtures.

In a preliminary experiment, the method of density gradient centrifugation could separate the lipid-protein complex from the unbound protein. As seen in Fig. 11, the majority of the activity of free ^{125}I-labeled 36 K protein was found near the top of the density gradient by this centrifugation. When the ^{125}I-36 K protein was mixed with unlabeled lipid mixture 1, the ^{125}I-activity was found to be as a peak at a higher density than the free ^{125}I-36 K protein. On the other hand, when the unlabeled protein was mixed with lipid mixture 1 containing ^{14}C-dipalmitoyl phosphatidylcholine the peak of ^{14}C-activity was found at the same higher density (data not shown). Therefore, both radioactivity peaks appear to indicate the lipid-protein complex formed using the protein and the lipid mixture.

The 18,000×g pellet mentioned above was then suspended in buffer A (0.5 m*l*), poured over density gradient, and centrifuged. Fig. 12 shows distribution profiles of ^{125}I-and ^{14}C-activities in the density gradients of the samples obtained from each lipid mixture and protein. The majority of both activities in the density gradients was found at the same peak, though appreciable amounts of unbound ^{125}I-activity were found near the top of the gradient. The molar ratio of dipalmitoyl phosphatidylcholine to 36 K protein in the lipid-protein complex (Fr. No. 9-14), calculated from the specific activities of the protein and dipalmitoyl phosphatidylcholine, is shown in Table 6. The dipalmitoyl phosphatidylcholine/36 K protein (mole/mole) ratio was about 1,200-1,300 in mixtures 1 and 2, while in mixtures 3 and 4 it was about 600. These results demonstrate that the presence of phosphatidylglycerol and cholesterol in the liposomes may be preferentially needed to form the lipid-protein complex, and the unsaturated phosphatidylcholine may not

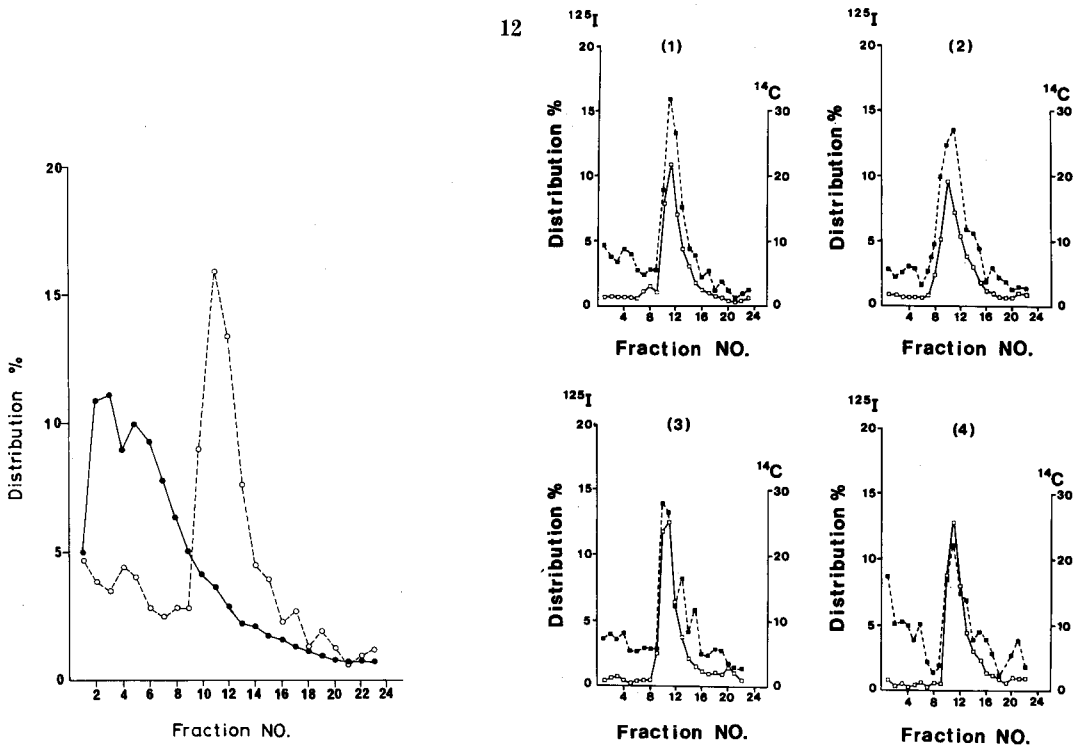

Fig. 11 Distribution of radioactivity of ^{125}I-36 K protein in sucrose density gradients. The percent distribution was calculated from total radioactivities recovered from the tubes.
●——●: ^{125}I-radioactivity in an experiment using ^{125}I-36 K protein alone without any of lipid mixtures
○---○: ^{125}I-radioactivity in an experiment using ^{125}I-36 K protein with lipid mixture 1, the composition of which is shown in Table 1.

Fig. 12 Distribution of radioactivities of ^{125}I-36 K protein and ^{14}C-dipalmitoyl phosphatidylcholine in sucrose density gradients in experiments using various lipid mixtures. The percent distribution was calculated from total radioactivities recovered from the tubes.
■——■: ^{125}I-radioactivity of 36 K protein
□——□: ^{14}C-radioactivity of dipalmitoyl phosphatidylcholine
(1): lipid mixture 1
(2): lipid mixture 2
(3): lipid mixture 3
(4): lipid mixture 4
Each lipid mixture contained ^{14}C-dipalmitoyl phosphatidylcholine. The composition of lipid mixtures is shown in Table 1.

be an essential component for the binding of 36 K protein to the liposomes.

Furthermore, effects of phosphatidylglycerol in the binding of the 36 K protein to the liposomes were studied in terms of its molecular stecies classified according to the degree of unsaturation. These experiments were carried out using mixtures 1 and 2. The results are shown in Table 7. It was noted that there were observed no significant differences in the molar ratio of dipalmitoyl phosphatidylcholine to 36 K protein in the lipid-protein complex among the molecular species of phosphatidylglycerol.

Table 6 *Radioactivity found in lipid-protein recombinant formed by ^{125}I-36 K protein and various lipid mixtures containing ^{14}C-dipalmitoyl phosphatidylcholine.*

Lipid mixture	^{125}I-36 K protein (Fr. No. 9-14)*		^{14}C-DPPC (Fr. No. 9-14)		DPPC/36 K protein	
	Radioactivity	Amount**	Radioactivity	Amount***	nmole/μg	mole/mole
1	9,511 dpm	9.15 μg	11,951 dpm	344.6 nmole	37.7	1,355
2	9,181	8.83	9,020	297.0	33.6	1,210
3	8,885	8.54	4,675	143.6	16.8	605
4	7,087	6.61	3,778	108.9	16.5	593

* Fr. No. 9-14 represents fraction tubes in density gradient centrifugation which contain lipid-protein recombinant

** The amount was calculated on the basis of specific activity of 36 K protein (1,040 dpm/μg).

*** The amount was calculated on the basis of specific activities of DPPC (mixture 1, 34,682; mixture 2, 30,373; mixture 3, 32,558; mixture 4, 34,682 dpm/μmole).

DPPC: dipalmitoyl phosphatidylcholine.

Table 7 *Effects of molecular species of phosphatidylglycerol in the recombination of 36 K protein and dipalmitoyl phosphatidylcholine.*

Lipid mixture	Molecular species of phosphatidylglycerol	36 K protein (μg)	DPPC (nmole)	DPPC/36 K protein (mole/mole)
1	Dipalmitoyl	10.5 (42.0)	390 (61.9)	1,339
	Oligoenoic	10.5 (42.0)	424 (67.3)	1,455
	Tetraenoic	5.6 (22.4)	186 (29.5)	1,192
2	Dipalmitoyl	6.9 (27.6)	288 (37.4)	1,526
	Oligoenoic	10.0 (40.0)	368 (47.3)	1,325
	Tetraenoic	8.2 (32.8)	290 (37.7)	1,273

The amounts of 36 K protein and DPPC were obtained from the specific activities of ^{125}I-36 K protein and ^{14}C-DPPC. The values in parentheses represent the recovery of 36 K protein and DPPC in the lipid-protein recombinant. Oligoenoic species represents a mixture of monoenoic and dienoic species. The composition of lipid mixturen 1 and 2 are shown in Table 1.

DPPC: dipalmitoyl phosphatidylcholine.

4 Discussion

Present analytical data on broncho-alveolar washings from patients with alveolar proteinosis clearly demonstrate that there are marked differences in the biochemical compositions of the supernatant and precipitate fractions. The precipitate fraction, i. e., water-insoluble materials, contains lipids as the major component, the majority of which is dipalmitoyl phosphatidylcholine, which is known to be a main component of the pulmonary surfactant[33]. It is known that dipalmitoyl phosphatidylcholine is contained not only in lung surfactant-related fractions but also in the cell membranes of lung tissues[34,35]. However, the ratio of phospholipid to protein in the precipitate fraction was very similar to that in alveolar wash of rat lung. Moreover, Akino *et al.*[6] reported that the ratios of protein to DNA and phospholipid to DNA observed in the precipitate fraction were significantly higher than those in rat lung parenchyma. These facts suggest that the materials accumulated in this disease are derived mainly from lung surfactant-related fractions of the lung, and not from cells and debris which are known also to be accumulated in the alveoli. The protein of the precipitate fraction exists in small amounts, but mainly comprises two glycoproteins

which are 62 K and 36 K proteins. One of these proteins, i. e., 36 K protein, seems to be the same protein as known to be contained in the intra- and extra-cellular surfactant fraction of normal lung[36,37]. Bhattacharyya *et al.*[10] reported that glycoproteins isolated from the pulmonary washings of rabbits were identical in molecular weights, amino acid and carbohydrate compositions with those found in pulmonary washings of patients with alveolar proteinosis. The present study also demonstrated that antibodies specific for 36 K protein isolated from the pulmonary washings from patients with this disease reacted intensely with lung surfactant apoproteins of human and pig, when dot-immunobinding assay method was employed, indicating that the 36 K protein might be contained in lung surfactant apoproteins. These analytical findings on lipid and protein profiles of the sedimented materials strongly support the idea that the materials accumulated in the alveolar space in this disease are derived mainly from the intra- and extra-cellular surfactant fractions, i. e., the materials existing normally in the alveoli may accumulate excessively in the alveoli of patients with alveolar proteinosis, probably due to the disturbance of the turnover of lung surfactant in the alveoli.

The present study demonstrated that total amounts of protein and phospholipid accumulated in the alveoli of a right or left lung of the patients were about 17 g and about 2.3 g, respectively. Although the control values of protein and phospholipid present in the alveoli of the normal human lung have not been determined, those can be roughly calculated to be about 0.3 g for protein and 0.15 g for phospholipid based on the data from rat lung washings[18]. Namely, the accumulation of protein and phospholipid in the alveolar space of patients with alveolar proteinosis might be about 43-fold and 8-fold of the normal state, respectively. These accumulations occurred from six months to two years in most cases. The turnover of alveolar phosphatidylcholine can be calculated to be about 1.1 g/day/a lung on the basis of its turnover rate (0.2 μmole/h/g wet tissue) of alveolar phosphatidylcholine of rat lung reported by Toshima *et al.*[38] The values of phosphatidylcholine accumulation in the alveoli of the patients with this disease was about 1.4 g for a lung. Only 1.3 days might be need for this accumlation. Therefore, these findings suggest that the disturbance of turnover of alveolar phosphatidylcholine may not be so severe, and the accumulation of phosphatidylcholine in the alveoli may occur slowly during long time of periods in this disease.

The cellular source of this particulate material or the cause of its accumulation remains unknown. Overproduction of lung surfactant and increased secretion of lamellar organelles into the alveoli have been implicated in alveolar proteinosis[39]. Evidence has also been presented that alveolar clearance is delayed with the prolonged retention of both lipid and protein in this disease[2]. This fact suggests that impairment of alveolar clearance is an important pathogenic factor in this disease. The mechanism of the removal or degradation of phospholipids in the alveoli remains unknown, although several possibilities have been reported. In this respect, Schober *et al.*[40] reported that the intra-alveolar material in alveolar proteinosis appears to originate from the ingested lamellar bodies in lysosomes of alveolar macrophages. They suggested that pulmonary alveolar proteinosis represents a state in which the catabolic capacities of alveolar macrophages have been overstressed. However, it is still unknown whether the abnormalities in macrophages are the primary defect of the secondary phenomenon of the disease.

In the present study, the 36 K protein was isolated from patient's lavages in relatively large scales. Although the 36 K protein was not identified completely to be lung surfactant apoproteins, it was strongly suggested from the present analytical and immunological studies that the protein is a constituent of lung surfactant apoproteins. It should also be noted that the 36 K protein was isolated in the presence of a sulfhydryl reducing reagent. When the sedimented materials did not react with 2-mercaptoethanol prior to gel filtration, the great majority of the proteins were eluted in the void volume. Namely, the presence of the sulfhydryl reducing reagent was essential for the isolation of the 36 K protein. Moreover, Bhattacharyya and Lynn[41] reported that two glycoproteins of 62,000 and 36,000 molecular weights were proteolytic products of native high molecular weight proteins, i. e., 80,000 or 200,000 molecular weight protein. These

facts indicate that the 36 K protein isolated in this study may be a subunit of high molecular weight glycoproteins which probably are native forms of lung surfactant apoproteins. On the other hand, King and Martin[42] reported that 36 K protein is secreted from alveolar type II cells into the alveolar lumen with the surfactant lipids. In the alveolar lumen, it is likely that this protein may interact with the lipids to stabilize structural transformation of lung surfactant complex and to modulate the physiological action of lung surfactant. Therefore, in this study the interaction of lipid and protein was investigated using the 36 K protein isolated from patient's lavages, in the assumption that this protein ma be a subunit of lung surfactant apoproteins.

The ratio of lipid to protein used in the present reconstitution studies was about 32 by weight, when assumed that the average molecular weight of lipids is 800. This value was calculated from the data of the pulmonary surfactant which was highly purified from dog lung washings by Clements *et al.*[43] They showed that the protein represents 5% by weight of surfactant and that the others are lipids. Accordingly, the ratio of lipid to protein in the highly purified surfactant was about 19 (w/w). Assuming that the protein of 36,000 molecular weight may comprise about 60% of the surfactant apoprotein, the ratio (w/w) of lipid to protein would be about 32. This ratio by weight corresponds to the molar ratio of 1440.

The concentration of lipids in the experiments of interaction with 36 K protein was usually 0.5 mM. The concentrations of dipalmitoyl phosphatidylcholine and egg phosphatidylcholine were calculated to be 0.32 to 0.43 mM and about 0.1 mM, respectively. These values are much greater than the critical micellar concentrations of dipalmitoyl phosphatidylcholine (4.6×10^{-10}M)[44] and egg phosphatidylcholine (5×10^{-7} M)[44]. The interaction of lipids to protein was carried out at 37° C. The following experimental procedures were carried out at 20–25° C. These temperatures are below the phase transition temperature of dipalmitoyl phosphatidylcholine (41° C)[45]. Therefore, the lipid mixtures containing dipalmitoyl phosphatidylcholine used in the present study were likely to exist as multivesicular liposomes, though morphology of the liposomes was not examined.

The 36 K protein bound with liposomes showed one peak in the density gradient by the centrifugation. Since unbound protein was distributed near the top of the density gradient, it is no doubt that the 36 K protein could bind with the multivesicular liposomes of different lipid mixtures. The possible mechanism by which the 36 K protein binds with lipids in a large molar ratio of lipid to protein, is the insertion of the glycoprotein into the lamellar of the multilamellar liposomes.

The main purpose of this study was to examine whether the particular constituent affects the binding between the lipid and 36 K protein, and especially to obtain some insight on the role of phosphatidylglycerol which uniquely occurs in pulmonary surfactant[46]. Present results demonstrated that the presence of phosphatidylglycerol and cholesterol in the lipid mixtures displayed enhancing effects on the binding of liposomes to the 36 K protein, resulting in an increase in the molar ratio of lipid to protein. The unsaturated phosphatidylcholines which occur about 5% in highly purified pulmonary surfactant, may not be essential components for the liposomes bound with the 36 K protein. Phosphatidylglycerol in pulmonary washings contains about 20% of saturated species in which dipalmitoyl species is predominant[47]. The present study demonstrated that there were no significant differences in the binding of liposomes to the 36 K protein among molecular species of phosphatidylglycerol. It appears that dipalmitoyl species of phosphatidylglycerol is not more effective than other species in the forming of a lipid-protein complex. Further studies will be needed to elucidate the physiological significance of disaturated phosphatidylglycerol in the surfactant system.

Acknowledgments

We thank the following physicians for providing broncho-alveolar washings: Dr. Ogawa, H., Department of Anesthesiology, Asahikawa Medical College; Dr. Nakamura, M., Department of Internal Medicine, Sapporo Medical College; Dr. Arai, T., Division of Respiratory Disease, Department of Internal Medicine, Jichi Medical School; Dr. Kamishima, K., Department of Internal Medicine, Hokkaido University Medical School.

References

1. Rosen, S. H., Castleman, B. and Liebow, A. A.: A pulmonary alveolar proteinosis. N. Engl. J. Med. **258,** 1123-1142 (1958).
2. Remirez-R, J. and Harlan, W. R.: Pulmonary alveolar proteinosis. Amer. J. Med. **45,** 502-512 (1968).
3. Ramirez-R, J., Schwartz, B., Dowell, A. R. and Lee, S. D.: Biochemical composition of human pulmonary washing. Arch. Intern. Med. **127,** 395-400 (1971).
4. McClenahan, J. M. and Mussenden, R.: Pulmonary alveolar proteinosis. Arch. Intern. Med. **133,** 284-287 (1974).
5. Sahu, S., DiAugustine, R. P. and Lynn, W. S.: Lipids found in pulmonary lavage of patients with alveolar proteinosis and in rabbit lung lamellar organelles. Amer. Rev. Resp. Dis. **114,** 177-185 (1976).
6. Akino, T., Okano, G. and Ohno, K.: Alveolar phospholipids in pulmonary alveolar proteinosis. Tohoku J. Exp. Med. **126,** 51-62 (1978).
7. Onodera, T., Nakamura, M., Sato, T. and Akino, T.: Biochemical characterization of pulmonary washings of patients with alveolar proteinosis, interstitial pneumonitis and alveolar cell carcinoma. Tohoku J. Exp. Med. **139,** 245-263 (1983).
8. Passero, M. A., Tye, R. W., Kilburn, K. H. and Lynn, W. S.: Isolation and characterization of two glycoproteins from patients with alveolar proteinosis. Proc. Natl. Acad. Sci. USA **70,** 973-976 (1973).
9. Bhattacharyya, S. N. and Lynn, W. S.: Studies on structural relationship between two glycoproteins isolated from alveoli of patients with alveolar proteinosis. Biochim. Biophys. Acta **494,** 150-161 (1977).
10. Bhattacharyya, S. N., Passero, M. A., DiAugustine, R. P. and Lynn, W. S.: Isolation and characterization of two hydroxyproline-containing glycoproteins from normal animal lung lavage and lamellar bodies. J. Clin. Invest. **55,** 914-920 (1975).
11. Frosolono, M. F., Charms, B. L., Pawlowski, R. and Slivka, S.: Isolation, characterization, and surface chemistry of a surface-active fraction from dog lung. J. Lipid Res. **11,** 439-457 (1970).
12. King, R. J. and MacBeth, M. C.: Physicochemical properties of dipalmitoyl phosphatidylcholine after interaction with an apolipoprotein of pulmonary surfactant. Biochim. Biophys. Acta **557,** 86-101 (1979).
13. Arvidson, G. A. E.: Structural and metabolic heterogeneity of rat liver glycerophosphatides. Eur. J. Biochem. **4,** 478-486 (1968).
14. Yang, S. F.: Phospholipase D from savoycabbage. Methods Enzymol. **14,** 208-211 (1969).
15. Bligh, E. G. and Dyer, W. J.: A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37,** 911-917 (1959).
16. Poorthuis, J. H. M., Yazaki, P. J. and Hostetler, K. Y.: An improved two dimensional thin-layer chromatography system for the separation of phosphatidylglycerol and its derivatives. J. Lipid Res. **17,** 433-437 (1976).
17. Akino, T., Abe, M. and Arai, T.: Studies on the biosynthetic pathways of molecular species of lecithin by lung slices. Biochim Biophys. Acta **248,** 274-181 (1971).
18. Toshima, N. and Akino, T.: Alveolar and tissue phospholipids of rat lung. Tohoku J. Exp. Med. **108,** 253-263 (1972).
19. Morrison, W. R. and Smith, L. M.: Preparation of fatty acid methylesters and dimethylacetales from lipids with boron fluoride-methanol. J. Lipid Res. **5,** 600-608 (1964).
20. Renkonen, O.: Individual molecular species of phospholipids. III. Molecular species of ox-brain lecithins. Biochim. Biophys. Acta **125,** 288-309 (1966).
21. Okano, G., Kawamoto, T. and Akino, T.: Comparison of molecular structure of glycerolipids in rat lung. Biochim. Biophys. Acta. **528,** 385

-393 (1978).
22. Bartlett, W. C.: Phosphorus assay in column chromatography. J. Biol. Chem. **234,** 466-468 (1959).
23. Van Handel, E. and Zilversmit, D. B.: Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med. **50,** 152-157 (1957).
24. Lowry, D. H., Rosebrough, N. J., Farr, A. L. and Randall, R. T.: Protein measurements with the folin phenol reagent. J. Biol. Chem. **193,** 265-275 (1951).
25. Weber, K. and Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244,** 4406-4412 (1969).
26. Zacharius, R. M. and Zell, T. E.: Glycoprotein staining following electrophoresis on acrylamide gel. Anal. Biochem. **30,** 148-152 (1969).
27. Hunkapiller, M. W., Lujan, E., Ostrander, F. and Hood, L. E.: Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91,** 227-257 (1983).
28. Hawkes, R., Niday, E. and Gordon, J.: A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119,** 142-147 (1982).
29. Esen, A., Conroy, J. M. and Wang, S-z.: A simple and rapid dot-immunobinding assay for zein and other prolamins. Anal. Biochem. **132,** 462-467 (1983).
30. Hunter, W. M. and Greenwood, F. C.: Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature **194,** 495-496 (1962).
31. King, R. J. and MacBeth, M. C.: Interaction of the lipid and protein components of pulmonary surfactant. Role of phosphatidylglycerol and calcium. Biochim. Biophys. Acta **647,** 159-168 (1981).
32. Spackman, D. H.: Accelerated methodsd. Methods Enzymol. **11,** 3-15 (1967).
33. Goerke, J.: Lung surfactant. Biochim. Biophys. Acta **344,** 241-261 (1974).
34. Nakamura, M., Onodera, T. and Akino, T.: Characteristics of phospholipids in human lung carcinoma. Lipids **15,** 616-623 (1980).
35. Honda, Y. and Nakamura, M.: A Comparative study on the phospholipid profiles of subcellular fractions isolated from human primary lung carcinoma and normal lung tissues. Sapporo Med. J. **52,** 613-626 (1983).
36. King, R. J., Klass, D. J., Gikas, E. G. and Clements, J. A.: Isolation of the apoproteins from canine surface-active material. Am. J. Physiol. **224,** 788-795 (1973).
37. Sawada, H., Yamabayashi, H. and Okajima, Y.: Isolation of apoprotein from bovine lung surfactant material by sodium deoxycholate. Biochim. Biophys. Acta **493,** 196-200 (1977).
38. Toshima, N., Akino, T. and Ohno, K.: Turnover time of lecithin in lung tissue and alveolar wash of rat. Tohoku J. Exp. Med. **108,** 265-277 (1972).
39. Larson, R. K. and Gordinier, R.: Pulmonary alveolar proteinosis: report of six cases, review of literature, and formulation of a new theory. Ann. Intern. Med. **62,** 292-313 (1965).
40. Schober, R., Bensch, K. G., Kosek, J. C. and Northway, W. H.: On the origin of the membranous intraalveolar material in pulmonary alveolar proteinosis. Exp. Mol. Pathol. **21,** 246-258 (1974).
41. Bhattacharyya, S. N. and Lynn, W. S.: Structural characterization of a glycoprotein isolated from alveoli of patients with alveolar proteinosis. J. Biol. Chem. **254,** 5191-5198 (1979).
42. King, R. J. and Martin, H.: Intracellular metabolism of the apoproteins of pulmonary surfactant in rat lung. J. Appl. Physiol. **48,** 812-820 (1980).
43. Clements, J. A. and King, R. J.: Composition of surface-active material. in The Biochemistry Biochemical Basis of Pulmonary Function, ed. by Crystal, R. G., (Dekker, New York), pp. 363-387 (1976).
44. Smith, R. and Tanford, C.: The critical micelle concentration of L-α-dipalmitoylphosphatidylcholine in water and water/methanol solution. J. Mol. Biol. **67,** 75-83 (1972).
45. Tsong, T. Y. and Kanehisa, M. I.: Relaxation phenomena in aqueous dispersions of synthetic lecithins. Biochemistry **16,** 2674-2680 (1977).
46. Pfleger, R. C., Henderson, R. J. and Waide, J.: Phosphatidylglycerol a major component of pulmonary surfactant. Chem. Phys. Lipids **9,** 51-68 (1972).
47. Okano, G. and Akino, T.: Variations in the molecular species of lung phosphatidylglycerol. Lipids **14,** 541-546 (1979).

Address for reprint requests:
Tsugutami Onodera

Department of Internal Medicine (Section 3), Sapporo Medical College South 1, West 17, Chuo-ku, Sapporo, 060, Japan

肺胞蛋白症患者肺胞腔に蓄積する物質の生化学的特徴

小野寺 次 民
札幌医科大学内科学第3講座 (主任 鈴木明 教授)

佐 藤 秀 紀 長 田 洋 子
札幌医科大学生化学第1講座 (主任 秋野豊明 教授)

5人の肺胞蛋白症患者から得た計15回の気管支肺胞洗浄液を約500×g，10分遠心して上清画分と沈渣画分に分離し，その脂質および蛋白質の分析を行ない，本症における肺胞腔に蓄積する物質の性状を検討した．上清画分は主として血清蛋白質から成る多量の蛋白質を含むが，脂質成分は少なかった．これに対し，沈渣画分，すなわち水不溶性物質では脂質が主成分であり，その大部分は肺サーファクタントの主要構成物として知られるdipalmitoyl phosphatidylcholineであった．水不溶性物質中，蛋白質は量的に少ないが，特徴ある蛋白質，すなわち主として分子量36,000 (36 K) と62,000 (62 K) の蛋白質を含んでいた．これら2種の蛋白質は糖蛋白であり，また比較的高率に疎水性アミノ酸を含んでいた．肺胞蛋白症患者の肺胞洗浄液から分離された36 K蛋白は，ヒトおよびブタの肺表面活性物質と同一の抗原性を有し，さらに肺組織にのみ局在することから，肺サーファクタントアポ蛋白と考えた．これらの分析所見から，肺胞蛋白症患者肺胞腔に蓄積する物質は細胞内および細胞外サーファクタント画分に由来することが強く示唆される．

さらに，36 K蛋白が肺サーファクタントアポ蛋白のsubunitであると仮定し，36 K蛋白と各種脂質との相互作用を検討した．その結果，dipalmitoyl phosphatidylcholineを含む脂質中にphosphatidylglycerolとcholesterolが存在した場合，リポソームと蛋白質との結合が増加した．つまり，36 K蛋白に対するdipalmitoyl phosphatidylcholineのモル比が増加した．しかし，phosphatidylglycerolの分子種間の相違による差は認められなかった．